Practitioner's Docket No. 60319-010

CHAPTER II

Preliminary Classification:

Proposed Class:

Subclass:

NOTE: *"All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P., § 601, 7th ed.*

TRANSMITTAL LETTER TO THE UNITED STATES ELECTED OFFICE (EO/US)

(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)

PCT/GB99/00071	08 January 1999	08 January 1998
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED

USE OF MASS FINGERPRINTING FOR IDENTIFICATION OF PROTEIN AFFINITY LIGANDS
TITLE OF INVENTION

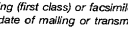
Stephen Roy Pennington
APPLICANT(S)

Box PCT
Assistant Commissioner for Patents
Washington D.C. 20231
ATTENTION: EO/US

CERTIFICATION UNDER 37 C.F.R. § 1.10*
(Express Mail label number is mandatory.)
(Express Mail certification is optional.)

I hereby certify that this Transmittal Letter and the papers indicated as being transmitted therewith is being deposited with the United States Postal Service on this date July 7, 2000, in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EK393504911US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Donna Crumit
(type or print name of person mailing paper)



Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

***WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,435, at 56,442.*

NOTE: To avoid abandonment of the application, the applicant shall furnish to the USPTO, not later than 20 months from the priority date: (1) a copy of the international application, unless it has been previously communicated by the International Bureau or unless it was originally filed in the USPTO; and (2) the basic national fee (see 37 C.F.R. § 1.492(a)). The 30-month time limit may not be extended. 37 C.F.R. § 1.495.

WARNING: *Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. § 1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing—See 37 C.F.R. § 1.8.*

NOTE: Documents and fees must be clearly identified as a submission to enter the national state under 35 U.S.C. § 371 otherwise the submission will be considered as being made under 35 U.S.C. § 111. 37 C.F.R. § 1.494(f).

I. Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. § 371:

a. ☒ This express request to immediately begin national examination procedures (35 U.S.C. § 371(f)).

b. ☒ The U.S. National Fee (35 U.S.C. § 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

2. Fees

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
☒*	TOTAL CLAIMS	18 −20=	0	× $18.00=	$
	INDEPENDENT CLAIMS	6 −3=	3	× $78.00=	234.00
	MULTIPLE DEPENDENT CLAIM(S) (if applicable)		3	+ $260.00	780.00
BASIC FEE**	☐ U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where an international preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: ☐ and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(1) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 C.F.R. § 1.492(a)(4)) ..... $96.00 ☐ and the above requirements are not met (37 C.F.R. § 1.492(a)(1)) ..... $670.00 ☐ U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY Where no international preliminary examination fee as set forth in § 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: ☐ has been paid (37 C.F.R. § 1.492(a)(2)) ..... $690.00 ☐ has not been paid (37 C.F.R. § 1.492(a)(3)) ..... $970.00 ☒ where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 C.F.R. § 1.492(a)(5)) $840.00				840.00
	Total of above Calculations				= 1854.
SMALL ENTITY	Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (note 37 C.F.R. § 1.9, 1.27, 1.28)				− 927
	Subtotal				927
	Total National Fee				$ 927
	Fee for recording the enclosed assignment document $40.00 (37 C.F.R. § 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".				40.00
TOTAL	Total Fees enclosed				$ 967.00

*See attached Preliminary Amendment Reducing the Number of Claims.

i. ☐ A check in the amount of _____ to cover the above fees is enclosed.

ii. ☒ Please charge Account No. 04-2223 in the amount of $ 967.00. A duplicate copy of this sheet is enclosed.

***WARNING:** *"To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date: * * * (2) the basic national fee (see § 1.492(a)). The 30-month time limit may not be extended." 37 C.F.R. § 1.495(b).*

WARNING: *If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C.F.R. § 1.495(b)(2). The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 apply to the period which is set. Notice of Jan. 3, 1993, 1147 O.G. 29 to 40.*

3. ☒ A copy of the International application as filed (35 U.S.C. § 371(c)(2)):

NOTE: Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment. "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36. See item 14c below.

a. ☒ is transmitted herewith.

b. ☐ is not required, as the application was filed with the United States Receiving Office.

c. ☐ has been transmitted

i. ☐ by the International Bureau.
Date of mailing of the application (from form PCT/1B/308): _____.

ii. ☐ by applicant on _____.
Date

4. ☒ A translation of the International application into the English language (35 U.S.C. § 371(c)(2)):

a. ☐ is transmitted herewith.

b. ☒ is not required as the application was filed in English.

c. ☐ was previously transmitted by applicant on _____.
Date

d. ☐ will follow.

5. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3)):

NOTE: The Notice of January 7, 1993 points out that 37 C.F.R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that: "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected." 1147 O.G. 29-40, at 36.

a. ☒ are transmitted herewith.

b. ☐ have been transmitted

i. ☐ by the International Bureau.
Date of mailing of the amendment (from form PCT/1B/308): _____.

ii. ☐ by applicant on (date) _____.
Date

c. ☐ have not been transmitted as

i. ☐ applicant chose not to make amendments under PCT Article 19. Date of mailing of Search Report (from form PCT/ISA/210.): _____.

ii. ☐ the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6. ☒ A translation of the amendments to the claims under PCT Article 19 (38 U.S.C. § 371(c)(3)):

a. ☐ is transmitted herewith.

b. ☒ is not required as the amendments were made in the English language.

c. ☐ has not been transmitted for reasons indicated at point 5(c) above.

7. ☒ A copy of the international examination report (PCT/IPEA/409)

☒ is transmitted herewith.

☐ is not required as the application was filed with the United States Receiving Office.

8. ☒ Annex(es) to the international preliminary examination report

a. ☒ is/are transmitted herewith.

b. ☐ is/are not required as the application was filed with the United States Receiving Office.

9. ☒ A translation of the annexes to the international preliminary examination report

a. ☐ is transmitted herewith.

b. ☒ is not required as the annexes are in the English language.

10. ☒ An oath or declaration of the inventor (35 U.S.C. § 371(c)(4)) complying with 35 U.S.C. § 115

a. ☐ was previously submitted by applicant on _____. Date

b. ☒ is submitted herewith, and such oath or declaration

i. ☒ is attached to the application.

ii. ☐ identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. § 1.70.

c. ☐ will follow.

II. Other document(s) or information included:

11. ☒ An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):

a. ☒ is transmitted herewith.

b. ☐ has been transmitted by the International Bureau. Date of mailing (from form PCT/IB/308): _____.

c. ☐ is not required, as the application was searched by the United States International Searching Authority.

d. ☐ will be transmitted promptly upon request.

e. ☐ has been submitted by applicant on _____. Date

12. ☒ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98:

a. ☐ is transmitted herewith.

Also transmitted herewith is/are:

☐ Form PTO-1449 (PTO/SB/08A and 08B).

☐ Copies of citations listed.

b. ☒ will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. § 371(c).

c. ☐ was previously submitted by applicant on _____. Date

13. ☒ An assignment document is transmitted herewith for recording.

A separate ☒ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.

_____

_____

_____

14. ☒ Additional documents:

a. ☐ Copy of request (PCT/RO/101)

b. ☒ International Publication No. WO 99/35502

i. ☐ Specification, claims and drawing

ii. ☒ Front page only

c. ☒ Preliminary amendment (37 C.F.R. § 1.121)

d. ☐ Other

_____

_____

_____

15. ☒ The above checked items are being transmitted

a. ☒ before 30 months from any claimed priority date.

b. ☐ after 30 months.

16. ☐ Certain requirements under 35 U.S.C. § 371 were previously submitted by the applicant on _____, namely:

_____

_____

_____

_____

_____

AUTHORIZATION TO CHARGE ADDITIONAL FEES

WARNING: *Accurately count claims, especially multiple dependant claims, to avoid unexpected high charges if extra claims are authorized.*

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

NOTE: "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

☒ The Commissioner is hereby authorized to charge the following additional fees that may be required by this paper and during the entire pendency of this application to Account No. 04-2223.

☒ 37 C.F.R. § 1.492(a)(1), (2), (3), and (4) (filing fees)

WARNING: *Because failure to pay the national fee within 30 months without extension (37 C.F.R. § 1.495(b)(2)) results in abandonment of the application, it would be best to always check the above box.*

☒ 37 C.F.R. § 1.492(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action.

☒ 37 C.F.R. § 1.17 (application processing fees)

☒ 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a).

☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying . . . issue fee." From the wording of 37 C.F.R. § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

☐ 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).

SIGNATURE OF PRACTITIONER

Reg. No.: **31,843**

Tel. No.: (248) **203-0849**

Customer No.:

Robert L. Kelly

(*type or print name of practitioner*)

Dykema Gossett PLLC
39577 Woodward Avenue, Suite 300

P.O. Address

Bloomfield Hills MI 48304

60319-010

VERIFIED STATEMENT BY ASSIGNEE CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c))
Nonprofit Organization

I hereby declare that I am an official of **The University of Liverpool**, a nonprofit constitutional corporation of the City of Liverpool, Great Britain having its principal place of business at Senate House, Abercromby Square, Liverpool L69 3BX Great Britain, and that I am empowered to act on its behalf.

I hereby declare that the above-identified organization qualifies as a nonprofit organization as defined in 37 CFR 1.9(e)(1), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled: **USE OF MASS FINGERPRINTING FOR IDENTIFICATION OF PROTEIN AFFINITY LIGANDS**, by Inventor **Stephen Roy Pennington** described in the United States patent application filed herewith.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventors, who could not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under CFR 1.9(d): None.

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent to which this verified statement is directed.

Dated: 4 July 2000

By: D. B. SEWITSON
Title: SENIOR ASSISTANT REGISTRAR

Docket No. 60,319-010

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Stephen Roy Pennington

Serial No. PCT/GB99/00071 filed January 08, 1999

U.S. Serial No. Unassigned - filed herewith

For: USE OF MASS FINGERPRINTING FOR IDENTIFICATION OF PROTEIN AFFINITY LIGANDS

Assistant Commissioner For Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

Prior to examination and prior to calculating the filing fee, please amend the claims as follows:

Please amend claims 4, 5, 6, 7, 10, 11, 17 and 18 as follows:

4. A method as claimed in [any of the preceding claims] claim 1 wherein the one of more proteins of interest are present in a mixture of proteins.

5. A method as claimed in [any of the preceding claims] claim 1 wherein the method is a shotgun method for selecting and identifying protein affinity ligands to a plurality of proteins.

6. A method as claimed in [any of the preceding claims] claim 1 wherein the other mass spectrometry based characterisation includes acquisition of sequence tag data.

7. A method as claimed in [any of the preceding claims] claim 1 wherein the antibodies generated in step (B)(i.) are immobilised on a support comprising nitrocellulose or PVDF.

10. A method as claimed in [any of claims] claim 7 [to 9] wherein the eluting agent is a volatile reagent.

11. A method as claimed in claims 10, 22, 23, 24, 25, 26 or 27 wherein the volatile reagent is formic acid.

17. A method as claimed in [any of the preceding] claims 1, 2, 7, 8, 9, 10, 19, 20, 21, 22, 23, 24, 25, 26 or 27 wherein the peptide mass fingerprint is obtained by mass spectrometry.

18. A method as claimed in [any of the preceding] claims 1, 2, 7, 8, 9, 10, 19, 20, 21, 22, 23, 24, 25, 26 or 27 further comprising the use of automated well plate handling technology and automated high-throughput mass spectrometry.

Please add the following claims:

19. A method as claimed in claim 2 wherein the antibodies generated in step (B)(i.) are immobilised on a support comprising nitrocellulose or PVDF.

20. A method as claimed in claim 19 wherein the support upon which the antibodies are immobilised are treated with an oligosaccharide or polyvinylpyrrolidine solution to block any remaining binding sites.

21. A method as claimed in claim 20 wherein the oligosaccharide is ficoll.

22. A method as claimed in claim 8 wherein the eluting agent is a volatile reagent.

23. A method as claimed in claim 9 wherein the eluting agent is a volatile reagent.

24. A method as claimed in claim 10 wherein the eluting agent is a volatile reagent.

25. A method as claimed in claim 19 wherein the eluting agent is a volatile reagent.

26. A method as claimed in claim 20 wherein the eluting agent is a volatile reagent.

27. A method as claimed in claim 21 wherein the eluting agent is a volatile reagent.

28. A method as claimed in claim 2 wherein the one of more proteins of interest are present in a mixture of proteins.

29. A method as claimed in claim 2 wherein the method is a shotgun method for selecting and identifying protein affinity ligands to a plurality of proteins.

30. A method as claimed in claim 2 wherein the other mass spectrometry based characterisation includes acquisition of sequence tag data.

REMARKS

Consideration and allowance are respectfully requested.

Respectfully submitted,

Robert L. Kelly
Registration No. 31,843
DYKEMA GOSSETT PLLC
39577 Woodward, Ste. 300
Bloomfield Hills, MI 48304
(248) 203-0849
Attorneys for Applicant

Dated: July 7, 2000

CERTIFICATE OF MAILING (37 C.F.R. 1.10)

I hereby certify that this paper is being deposited with the United States Postal Service on this date: July 7, 2000, in an envelope as "Express Mail Post Office to Addressee," Mailing Label Number EK393504911US, and is part of the documents transmitted for entry in the U.S. National Phase under Chapter II, addressed to: Box PCT, Assistant Commissioner for Patents, Washington, D.C. 20231.

Donna M. Crumit

BH\259095
ID\ RLK:dc

DESCRIPTION

USE OF MASS FINGERPRINTING FOR IDENTIFICATION OF PROTEIN AFFINITY LIGANDS

The present invention relates to the field of proteome analysis (proteomics) and to methods for the generation of tools for proteomics and the use of these tools in for example protein expression.

The last few years have heralded an unprecedented increase in gene sequencing and identification as exemplified by the announcement of the complete genome sequence of prokaryotic and eukaryotic cells and organisms including the model eukaryote *C. elegans* (see http://www.nih.gov/news/pr/dec98/nhgri-09.htm; Science 11 Dec 1998). The sequencing of the human genome continues to accelerate with completion projected for 3-4 years time. This growing DNA sequence data is being used as a platform for the systematic investigation of gene (mRNA) expression and function. Such investigations combined with associated informatics are transforming basic biological research and will transform the drug discovery, development and delivery processes. However, it has become increasingly apparent that DNA sequence data in itself often provides little information about the function of the encoded protein products. Furthermore, measurement of gene expression at the mRNA level does not always give an accurate representation of the expression of the corresponding proteins nor indeed of the extent to which they may be post-translationally modified. Importantly, it is predominantly proteins that execute biological function. Hence, there is a growing desire to analyse, in a systematic and comprehensive manner, the expression and activity of the protein products of an organism's genome - this aim provides a working definition of proteome analysis (or proteomics) (see Pennington *et al.* Proteome analysis: from protein characterisation to biological function. Trends Cell Biol. 7, 168-173 and references therein). It is important to emphasise that the activity of individual proteins may be regulated by a number of

different mechanisms including their level of expression within individual tissues or cells, the extent and type of post-translational modifications, their subcellular localisation and their interactions with other proteins. Proteomics therefore encompasses a broad range of experimental approaches.

Existing approaches for proteomics are best exemplified by the use of two-dimensional electrophoresis (2-DE) to resolve and quantify the expression of several thousand proteins simultaneously with the application of a portfolio of methods to identify the resolved proteins. However, protein identification and full primary sequence determination still frequently rely on the application of existing DNA sequence data or of molecular biology based methods for identification of the corresponding gene sequence. Attempts are being made to develop alternative orthogonal approaches to high-throughput measurement of protein expression which, like 2-DE, are based on the initial separation of proteins from mixtures. It is notable however, that some of the most dramatic improvements in the measurement of mRNA expression have come from separation-independent methods that rely on *molecular recognition*. In summary, existing approaches for the measurement of protein expression have many inherent limitations - they are laborious, time consuming, suffer reproducibility problems, are not as sensitive as required and are unlikely to be readily applicable to the simultaneous analysis of all the proteins of even a 'simple' genome. Moreover, they have one overriding limitation - they do not result *per se* in the generation of experimental tools for measurement or manipulation of the proteins. So, for example, if 2-DE (or indeed any other existing protein based method) is used to identify a group of proteins associated with a particular disease (by differential analysis of protein expression) the process of identifying the proteins will not have led to the production of experimental tools. One example of such tools are molecules or ligands that can specifically bind to the individual proteins of interest with appropriate affinity for the desired application - 'protein affinity ligands'. Such tools have many uses. For example they might be used to measure protein expression, purify the protein

for functional analysis, influence the activity of the proteins or be used as diagnostic reagents. The invention described herein provides a high throughput method to produce and identify such 'protein affinity ligands' either to proteins of interest or to proteins that may previously have been unidentified. It is an aim of the present invention to provide a high throughput method to produce 'catalogued' libraries of 'protein affinity ligands'. These 'protein affinity ligands' have many potential applications and will have applicability in drug target identification and validation , drug discovery and development and as diagnostic tools. They will also provide important tools for the development of separation independent approaches to the measurement of protein expression and post-translational modification.

The term 'protein affinity ligand' used herein refers to a molecule (large or small) that binds to a protein be it the polypeptide backbone (or part thereof) or any modified part of the protein, for example, a phosphorylated, fatty acylated, ADP-ribosylated or glycosylated moiety or indeed any known or hitherto unidentified modification either natural or introduced by experimental intervention. Such protein affinity ligands may be best exemplified by antibodies, polyclonal, monoclonal or phage-displayed, but include peptide or peptoid moieties (including peptide-nucleic acid molecules), oligonucleotides, modified oligonucleotides or indeed any molecule or chemical that binds to a protein with appropriate specificity and affinity. Chemicals might include those selected from combinatorial libraries. Hence in one embodiment, the invention is directed towards antibodies as the 'protein affinity ligands', although other affinity ligands may be produced and identified by the invention.

Described herein is a method, termed "inverse screening",of identifying and isolating 'protein affinity ligands' to individual proteins. Also described herein is a method of using mixtures of proteins to generate libraries of well characterised 'protein affinity ligands'. It is a feature of the invention that 'protein affinity

ligands' to individual proteins may be generated without requiring access to the individual protein. The 'protein affinity ligands' so generated and identified by the present method and their protein targets (if previously unknown) are also therefore the subject of the present invention.

According to the present invention there is provided a method of selecting and / or identifying one or more protein affinity ligand's that bind to one or more proteins of interest comprising the steps of:

(A) obtaining a real or theoretical peptide mass fingerprint or other mass spectrometry based characterisation or other protein characterisation of the one or more proteins by either:

i. Subjecting the one or more proteins to peptide mass fingerprinting or other mass spectrometry based characterisation or other protein characterisation; or

ii. Predicting the peptide mass fingerprint or other mass spectrometry based characterisation or other protein characterisation from known data;

(B) utilising the one or more proteins either individually or as a mixture to:

i. Generate one or more antibodies thereto by immunisation ;and/or

ii. Select, using a single or multiple rounds of binding, one or more protein affinity ligands thereto;

(C) screening the one or more antibodies generated in step (B)(i) and/or the one or more protein affinity ligands selected in step (B)(ii) by:

i. adding the one or more proteins individually or as a mixture of proteins to the one or more antibodies generated in step (B)(i) or the one or more protein affinity ligands selected in step (B)(ii), each antibody or protein affinity ligand being used individually, and

ii. after removing any proteins which have not bound, eluting the at least one protein that has bound;

(D) subjecting the at least one eluted protein to peptide mass

fingerprinting and / or other mass spectrometry based characterisation and/or other protein characterisation ; and

(E) by comparing the peptide mass fingerprints or other mass spectrometry based characterisation or other protein characterisation obtained in steps (A) and (D) selecting and/or identifying the at least one protein affinity ligand that binds to the one or more proteins of interest.

The other protein characteristics might include, for example, amino acid composition analysis or other sensitive and specific techniques.

The other mass spectrometry based characteristics might include, for example, the use of sequence tag data.

The mixture of proteins referred to above may be simple or complex; they may be obtained from any organism, any type of tissue, cell or combinations thereof that may have been subjected to any treatment. The protein mixture may also be obtained from fractionation of an organism, tissue or cell such as secreted, membrane, cytoplasmic, organellar or nuclear proteins or fractions of proteins bearing modifications for example carbohydrate containing or phosphorylated proteins. The protein mixture may also be obtained by *in vitro* expression of cDNAs. The proteins may be native or may have been modified in any way, for example, by biotinylation or by addition of fluorescent or other chemical moieties.

The 'protein affinity ligands' may specifically recognise more than one protein for example by way of binding to proteins that interact with each other. Under these circumstances the mass spectrometry data may be used to reveal such multiple proteins and the 'protein affinity ligands' may have additional value and/or use by virtue of their ability to recognise (i) multiple proteins of, for example, a protein family, or (ii) proteins that are bound to each other through protein:protein interactions.

Preferably the one or more proteins of interest are resolved by 2D electrophoresis.

Preferably the antibodies obtained in step (B)(i) are cloned prior to step (C).

Preferably the antibodies are immobilised on a solid phase support. Preferably the support is of nitrocellulose or PVDF. Such supports were found to be particularly effective at binding proteins in the methods of the invention.

Preferably the remaining binding sites on the solid support, after the antibodies are immobilised thereon, are blocked to prevent non-specific protein binding. Traditional agents proved ineffective for the methodology of the invention. However oligosaccharides and polyvinylpyrrolidines proved particularly effective.

Preferably a volatile reagent i.e one which is readily removed by for example evaporation, is used as the eluting agent. Formic acid was found to be particularly favoured since it could be used with a wide range of antibodies and was sufficiently volatile to be easily removed. Its use had the advantage that subsequent manipulation steps to remove the eluting agent were avoided.

In one embodiment of the present invention there is provided a method of generating monoclonal antibodies to one or more targeted proteins comprising the steps of:

(a) resolving individual proteins from a protein mixture;

(b) subjecting the resolved protein(s) to peptide mass fingerprinting to obtain a peptide mass profile;

(c) utilising one or more of the resolved proteins to generate one or more monoclonal antibodies thereto by immunisation and generation of hybridomas;

(d) adding a protein mixture to the one or more antibodies generated in Step (c) to select those proteins which bind the one or more monoclonal antibodies, and subjecting the selected protein(s) to peptide mass fingerprinting to obtain a

peptide mass profile;

(e) comparing the data obtained in steps (b) and (d); and

selecting one or more hybridoma clones of interest.

In this embodiment step (b) may be omitted where the protein(s) of interest are previously known and DNA sequence data for the corresponding gene(s) exits as under these circumstances step (e) could be undertaken by comparing the data obtained in step (d) with that obtained from theoretical peptide mass fingerprinting of the corresponding DNA sequence.

In a second embodiment of the present invention there is provided a method of generating a library of antibodies comprising the steps of:

(a) resolving individual proteins from a protein mixture;

(b) subjecting the resolved protein(s) to peptide mass finger printing to obtain a peptide mass profile;

(c) utilising a protein mixture to generate one or more monoclonal antibodies thereto by immunisation and generation of hybridomas;

(d) adding a protein mixture to the one or more monoclonal antibodies generated in Step (c) to select to those proteins which bind the one or more monoclonal antibodies, and subjecting the selected protein(s) to peptide mass fingerprinting to obtain a peptide mass profile;

(e) comparing the data obtained in steps (b) and (d); and,

(f) identifying the monoclonal antibodies of potential interest for a monoclonal antibody library.

In this embodiment steps (a) and (b) may be omitted where the protein mixture derives from an organism for which significant DNA sequence data is available as under these circumstances step (e) could be undertaken by comparing the data obtained in step (d) with that obtained from theoretical peptide mass fingerprinting of the potential open reading frames encoded in the DNA sequence data.

In a third embodiment of the present invention there is provided a process for selecting desired members of an affinity ligand library comprising the steps of:

(a) resolving a protein mixture;

(b) subjecting the resolved protein(s) to peptide finger printing to obtain a peptide mass profile;

(c) utilising one or more of the resolved proteins to select one or more affinity ligands from a library;

(d) adding the protein mixture to the one or more affinity ligands generated in step (c) to select those proteins which bind the one or more affinity ligands, and subjecting the selected protein(s) to peptide mass fingerprinting to obtain a peptide mass profile;

(e) comparing the data obtained in steps (b) and (d); and

(f) selecting one or more affinity ligands of interest.

In this embodiment steps (a) and (b) may be omitted where the protein mixture derives from an organism for which significant DNA sequence data is available as under these circumstances step (e) could be undertaken by comparing the data obtained in step (d) with that obtained from theoretical peptide mass fingerprinting of the potential open reading frames encoded in the DNA sequence data.

In each embodiment the protein mixture used in step (d) may or may not be the protein mixture used in the prior steps.

Hence, the process represents a novel linkage between the resolution and identification by a protein characterisation, for example, mass spectrometry of individual proteins present in protein mixtures with the screening of affinity ligands by such means. The most significant advantage of the process is the provision of a method to generate and screen affinity ligands to proteins without requiring pure protein. To date, no other screening process which circumvents the need for pure protein has been described. Furthermore, the present invention

provides a method to generate large numbers of 'protein affinity ligands' for which the target proteins are identified without requiring access to the pure proteins.

The present invention includes provision for undertaking those steps in which proteins are selected by a 'protein affinity ligand' and subsequently analysed by, for example, mass spectrometry (i.e. step (d)) directly on the mass spectrometry targets or alternatively after some additional processing of 'protein affinity ligands' or the selected proteins prior to mass spectrometry. The present invention also includes provision for isolation and manipulation of 'protein affinity ligands', for example affinity isolation of antibodies from tissue culture supernatants, prior to use for inverse screening

A number of aspects of the invention are described in more detail, by way of example only, with reference to the following examples and flow diagrams.

Comparative Example

Current Approach

In a conventional approach to the generation of antibodies, an antigen or mixture of antigens is administered to mice, rats or other animal and the immune response to the antigen(s) is monitored by ELISA, dot blotting or, less frequently, Western blotting. Having identified animals which have produced an adequate immune response, monoclonal antibodies may be produced by taking the spleen cells from the animal and fusing them with cells of a myeloma cell line to generate hybridomas – immortalised cells which produce antibodies. The hybridoma cells are cloned, for example, by limiting dilution and the clones then screened by a range of different screening approaches to determine which cells are producing the desired antibodies. For effective selection of hybridomas the screening process must be robust, rapid and reliable because it is undertaken in parallel with the

initial culture of the hybridoma cells. There are three classes of screening strategy: antibody capture assays, antigen capture assays and functional screens. In general, the vast majority of screens are currently undertaken by antibody capture. In this approach the pure antigen bound to a solid surface is used to capture the antibodies and these are then detected with appropriate labelled anti-immunoglobulin antibodies. This process screens for antibodies to an individual protein antigen and requires significant quantities of purified protein. Antigen capture screens are rarely used unless the protein antigen is available in large quantities – this is because they require labeling of the antigen (often with radioisotopes i.e. ^{125}I).

Thus, although immunisation may require only small amounts of an individual protein antigen (often less than 1μg is appropriate) the screening process requires relatively large quantities of pure protein. Thus, in the context of current approaches to proteome analysis, where 2-DE is used to resolve thousands of proteins in sub-μg quantities existing methods for antibody production and isolation are unsuitable.

Another important feature of the existing methods is that those hybridoma clones or mixtures thereof that do not produce antibodies that recognise the target antigen are discarded. In the present invention the hybridomas or other 'protein affinity ligands' are cloned at an early stage and each antibody screened. By archiving the cloned hybridomas or other 'protein affinity ligands' it is possible to re-screen them under different conditions and against different protein mixtures.

The process described below provides a novel approach to the widespread production of antibodies or other 'protein affinity ligands' to proteins individually or in mixtures. The novel linkage between resolution of protein mixtures and the screening of affinity ligands by, for example, mass spectrometry provides a powerful approach to the generation of characterised 'protein affinity ligands'.

Example 1

Process directed towards generation of monoclonal antibodies to 'targeted proteins'

A protein mixture is separated by, for example, two dimensional electrophoresis (2-DE) or by any other means to provide individual 'target proteins'.

The proteins of interest are subjected to peptide mass fingerprinting or other mass spectrometry based method – this gives a characteristic peptide pattern and other data such as sequence tag that are unique to each of the proteins and it is this data that is exploited to select antibodies which are capable of binding the 'target proteins'.

Antibodies secreted from individual hybridoma clones are allowed to interact with a protein mixture containing the protein or proteins for which antibodies are desired. The appropriate antibodies will bind the target protein(s) of interest and the protein bound by each antibody may then be eluted and subjected to peptide mass fingerprinting or other mass spectrometry based analysis. The specificity and sensitivity of the mass spectrometry will allow the identification of the target protein(s) of interest and so reveal which hybridoma cells produce antibodies to the target protein(s).

This method is described more fully with reference to Fig. 1, which is a flow diagram of the method.

To generate monoclonal antibodies to protein(s) of interest, mammals, for example, rats or mice are immunised (Step C) with individual proteins recovered from, for example, 2-DE gels. An aliquot of the protein(s) may be subjected to peptide mass fingerprinting (Step B).

After fusing spleen cells with myeloma cell lines the resulting hybridomas are cloned, for example, by limiting dilution of the cells. The antibodies produced by individual clones or mixtures of clones are screened by mass spectrometry. Thus, monoclonal antibodies are recovered from the tissue culture media and immobilised to a solid phase support which may for example be a 96 well plate. To the immobilised monoclonal antibodies is added a protein mixture and the proteins which have bound to individual monoclonal antibodies eluted and subjected to peptide mass fingerprinting (Step D) or other mass spectrometry based characterisation. By comparing the data (Step E) obtained with the data for the proteins of interest it will be possible to select (Step F) those monoclonal antibodies which are specific for the proteins and hence isolate the relevant clones.

In a further improvement the process is streamlined by application of automated multi-well plate handling technology and automated high-throughput mass spectrometry for example by use of automated sample target loading so facilitating the screening of large numbers of individual cell clones.

Example 2

Process directed towards the generation of an antibody library

This process is described with reference to Fig. 2, which is a flow diagram of the method.

Mice/rats are immunised (Step C) with a mixture of proteins and the immune response may be screened by ELISA using the protein mixture. To establish the 'distribution' of antibodies generated – i.e. the coverage of the polyclonal library, the antiserum may be used for Western blotting for example, against 1-DE or 2-DE resolved proteins of the original protein mixture (Step A) or any other protein

mixture. For those animals which produce antibodies of the desired coverage the spleen cells are fused with a myeloma cell line to generate hybridomas and the antibodies produced by the total pool of hybridoma cells may be re-screened by, for example, Western blotting against the 1-DE or 2-DE resolved proteins. This may be used to confirm that suitable antibody producing hybridomas have been generated. The hybridoma mixture is then cloned for example by limiting dilution and antibodies immobilised, a complex mixture of proteins added, the proteins which have bound eluted (Step D) and screened by mass spectrometry. As with example 1 the data can be compared (Step E) and the clones of interest selected (Step F).

The key advantages of the approaches outlined above are:
purified protein is not required for screening;
monoclonal cell lines are relatively stable and can be archived;
antibodies of high affinity and appropriate selectivity will be produced; and
the protein mixture used for screening the antibodies can be presented to the antibodies in a form tailored to the likely end application i.e. the proteins may be presented in native or denatured form.

Example 3

Process for selecting phage displayed antibodies

This process is described with reference to Fig. 3, which is a flow diagram of the method.

Following separation of protein mixtures by, for example, 2-DE (Step A), the proteins are Western blotted (Step C) using an antibody phage library with detection of bound phage. Individual protein spots of interest and associated antibody displaying phage are excised from the Western blot, the phage eluted and

used to re-infect *E. coli*. Individual colonies of *E. coli* may be propagated (in 96 well plate format), phage antibody secretion induced with IPTG and individual antibodies (PhAbs) recovered. The PhAbs are then immobilised and a protein mixture added. Proteins which bind are then eluted (Step D) and characterised by peptide mass fingerprinting. As with Example 1 the data can be compared (Step E) and PhAbs which bind the protein(s) of interest will be selected (Step F).

The key advantages of the approach outlined above are:
it avoids the typical 4-5 rounds of phage selection but may also be used with any conventional phage selection process;
purified protein is not required for screening;
the method also reveals whether any PhAbs which bound to the protein during Western blotting are specific for other proteins in the protein mixture. Each screen will therefore produce PhAbs to at least the protein of interest and potentially others as well;
the phage can be archived to generate a stable source of PhAbs (Step F).
modification of the Western blotting selection conditions and the conditions under which protein mixture is added to and washed from individual PhAbs can be modified to isolate PhAbs of desired properties (i.e. affinity, avidity); and
relevant selected phage can be genetically modified to alter properties of PhAbs.

Example 4

Inverse screening a polyclonal antiserum to a 'target protein' - bovine serum albumin

The inverse screening method was used to screen a commercially available polyclonal antibody to bovine serum albumin (BSA) against a mixture of proteins (containing BSA). Polyclonal anti-BSA antibody (5-80μg; Sigma B-3759) in phosphate-buffered saline (PBS) was immobilised in the wells of 96 well

filtration plates that incorporated a high protein binding hydrophobic PVDF membrane (Millipore MultiScreen® Immobilon™-P filtration plates) by incubating the pre-wetted membranes with the antibody containing solution for 2h at room temperature. The membranes were pre-wetted according to manufacturers instructions; thus to each well was added 50µl of 50% ethanol and the membranes incubated for 1min at room temperature and after vacuum aspirating the wells each well was washed twice with 200µl of de-ionised water or PBS. After antibody binding the wells were washed extensively with PBS (6 x 0.35ml). It is clear that different conditions for the immobilisation of antibody may be used and many have been examined in detail. These other methods include use of different buffers, different incubation times and temperatures, different methods for removal of remaining antibody solutions (vacuum aspiration through membrane or direct removal) all of which are known to those of skill in the art. The method described here proved optimal for the antibody used in this example. Other methods described where appropriate were optimal for the other examples cited. The use of conventional 96 well plates or other solid phase supports commonly used in immunoassays did not have sufficient protein binding capacity for the immobilisation of antibody. This is important because sufficient protein has to be recovered from the antibodies for subsequent characterisation.. The use of a solid phase support, in this case PVDF, in a suitable format for high throughput application represents a significant inventive step and is critical for some of the examples cited herein.

The extent to which antibody binding had been effected was examined by several methods. Most routinely it was examined by determining the protein concentration (Bradford assay) of the antibody solution after it had been incubated with the wells. Under the conditions described greater than 95% of the antibody was immobilised when antibody solutions containing up to 40µg of antibody were added to each well. The maximum protein binding recorded (using solutions containing up to 80µg of antibody) was 44µg which is in close agreement with the

manufacturer's data.

It was reasoned that the ability to identify the proteins eluted from immobilised antibody would be critically dependent on the efficacy with which remaining protein binding sites on the solid phase support could be blocked to prevent subsequent non-specific protein binding. Thus, it seemed likely that non-specific proteins eluting with the antibody bound protein would significantly reduce the ability to obtain a peptide mass fingerprint suitable for matching. It was for this reason that a number of different blocking reagents and conditions were examined. Unfortunately, many of these reagents (detergents such as Tween and Triton, protein blocks etc.)were found to impair peptide mass fingerprinting by MALDI mass spectrometry. Consequently the applicant investigated a number of blocking reagents including polyvinylpyrrolidones and oligosaccharides that are not normally used in immunoassays. It was found that the quality of peptide mass fingerprinting data obtained from proteins eluted from antibody was significantly enhanced by use of these named blocking reagents, particularly ficoll . This represents an important inventive step. Thus, once antibody had been immobilised the remaining protein binding sites were blocked by incubation with 0.5% ficoll in PBS for 2h at room temperature. The wells were then washed extensively with PBS (6 x 0.35ml), incubated with elution reagent (0.5% formic acid) and washed again with PBS (6 x 0.35ml). A further method also found to be effective involved combining the ficoll block described above with dry blocking as recommended for the manufacturer for treatment of Immobilon™-P membranes when used in standard Western blotting applications. In this approach, the wells were dried by carefully removing all remaining solution and incubating in a desiccated environment at 37°C for 2h. The wells were then treated with 0.5% ficoll in PBS for 2h at room temperature, washed, treated with elution reagent and washed again as described above.

Having immobilised the antibody and blocked remaining protein binding sites on

the solid phase support the antibody is exposed to a protein mixture and specifically bound protein(s) eluted. Evidently, the elution reagents and conditions must be compatible with the subsequent protein characterisation method which in this case was peptide mass fingerprinting by MALDI mass spectrometry. A large number of elution conditions were investigated with attention focussed on those that would minimise subsequent manipulation steps and be compatible with MALDI mass spectrometry. The applicant discovered that formic acid was particularly effective in eluting protein from a wide range of antibodies and was sufficiently volatile that it could be readily removed from the eluted protein samples prior to MALDI mass spectrometry. Thus, in this example immobilised antibody was incubated with a protein mixture (0.5-1.0mg; soya milk proteins) containing the protein antigen (BSA, 0.015-1.0mg) for 2h at room temperature. The wells were washed with PBS (6 x 0.35ml) and bound protein eluted by incubation with 20-100µl of 0.5% formic acid for 10 minutes at room temperature. The elution reagent was then removed by evaporating the samples to dryness under vacuum in a rotary evaporator.

Eluted proteins were subjected to peptide mass fingerprinting by standard protocols (see Courchesne, P.L. and Patterson, S.D. (1999) 2-D Proteome Analysis Protocols [Link Ed.], *Methods in Molecular Biology* 112, 487-511. Humana Press). Thus, protein samples were subjected to enzymatic digestion (37°C, 18 hours) in 25mM ammonium bicarbonate, pH 7.8 containing 50ng of trypsin. Protein-doped crystals were prepared using α-cyano-4-hydroxycinnamic acid (HCCA) as a matrix. A saturated HCCA matrix solution (10mg/ml) was prepared in 70% MeCN/0.1% TFA and any undissolved matrix removed by centrifugation. An aliquot (0.3-0.5μl) of sample was mixed with an equal volume of matrix solution on the target and allowed to air dry. In some cases it proved efficacious to remove impurities by washing the crystals by the addition of 5μl of ice cold 0.1% TFA which was aspirated after a few seconds. Washing steps were repeated up to 3 times. Samples were subjected to MALDI-mass spectrometry on

a Lasermat 2000 (Thermo Bioanalysis) or TOFSPEC 2E (Micromass) and a representative mass spectrum shown in Figures 4A and 4B.

The predominant peptide masses from the spectrum shown in Figures 4A and 4B were selected 'blind' (after subtraction of peaks shown to originate from residual non-specific protein binding by use of an appropriate control - i.e. proteins recovered from wells in which no antibody had been immobilised) and used to search protein sequence databases. The peptide masses obtained in this way resulted in the identification of 3 matching proteins with BSA being identified as the 1st ranking protein well above the other two ranked proteins.

Example 5

Inverse screening a monoclonal antibody against a target protein - bovine serum albumin

The inverse screening method was used to screen a commercially available monoclonal antibody to bovine serum albumin (BSA) against a mixture of proteins (containing BSA). Monoclonal anti-BSA antibody (5-80μg; Chemicon) in phosphate-buffered saline (PBS) was immobilised in the wells of 96 well filtration plates that incorporated a high protein binding hydrophobic PVDF membrane (Millipore MultiScreen® Immobilon™-P filtration plates) by incubating the pre-wetted membranes with the antibody containing solution for 2h at room temperature. The membranes were pre-wetted according to manufacturers instructions; thus to each well was added 50μl of 50% ethanol and the membranes incubated for 1min at room temperature and after vacuum aspirating the wells each well was washed twice with 200μl of PBS. After antibody binding the wells were washed extensively with PBS (6 x 0.35ml). It is clear that different conditions for the immobilisation of antibody may be used and many have been examined in detail. These other methods include use of different buffers, different ...

incubation times and temperatures, different methods for removal of remaining antibody solutions (vacuum aspiration through membrane or direct removal) all of which are known to those of skill in the art. The method described here proved optimal for the antibody used in this example. Other methods described where appropriate were optimal for the other examples cited.

The extent to which antibody binding had been effective was examined by several methods. Most routinely it was examined by determining the protein concentration (Bradford assay) of the antibody solution after it had been incubated with the wells. Under the conditions described greater than 95% of the antibody was immobilised when antibody solutions containing up to 40μg of antibody were added to each well.

Once antibody had been immobilised the remaining protein binding sites were blocked by incubation with 0.5% ficoll in PBS for 2h at room temperature. The wells were then washed extensively with PBS (6 x 0.35ml), incubated with elution reagent (0.5% formic acid) and washed again with PBS (6 x 0.35ml). Again, a number of different blocking conditions may be used and have been examined and these are obvious to those of skill in the art. One method found to be effective involved combining the ficoll block described above with dry blocking as recommended for the manufacturer for treatment of Immobilon™-P membranes when used in standard Western blotting applications. In this approach, the wells were dried by carefully removing all remaining solution and incubating in a desiccated environment at 37°C for 2h. The wells were then treated with 0.5% ficoll in PBS for 2h at room temperature, washed, treated with elution reagent and washed again as described above.

The immobilised antibody was then incubated with a protein mixture (0.5-1.0mg; soya milk proteins) containing the protein antigen (BSA, 0.015-1.0mg) for 2h at room temperature. The wells were washed with PBS (6 x 0.35ml) and bound

protein eluted by incubation with 20-100µl elution reagent (0.5% formic acid) for 10 minutes at room temperature. The elution reagent was then removed and evaporated to dryness under vacuum in a rotary evaporator. Eluted proteins were subjected to peptide mass fingerprinting by standard protocols (see Courchesne, P.L. and Patterson, S.D. (1999) 2-D Proteome Analysis Protocols [Link Ed.], *Methods in Molecular Biology* 112, 487-511. Humana Press). Thus, protein samples were subjected to enzymatic digestion (37°C, 18 hours) in 25mM ammonium bicarbonate, pH 7.8 containing 50ng of trypsin. Protein-doped crystals were prepared using α-cyano-4-hydroxycinnamic acid (HCCA) as a matrix. A saturated HCCA matrix solution (10mg/ml) was prepared in 70% MeCN 0.1% TFA and any undissolved matrix removed by centrifugation. An aliquot (0.3-0.5µl) of sample was mixed with an equal volume of matrix solution on the target and allowed to air dry. In some cases it proved efficacious to remove impurities by washing the crystals by the addition of 5µl of ice cold 0.1% TFA which was aspirated after a few seconds. Washing steps were repeated up to 3 times. Samples were subjected to MALDI-mass spectrometry on a Lasermat 2000 (Thermo Bioanalysis) or TOFSPEC 2E (Micromass) and a representative mass spectrum shown in Figures 5A and 5B.

The predominant peptide masses from the spectrum shown in Figure 5A and 5B were selected 'blind' (after subtraction of peaks shown to originate from residual non-specific protein binding by use of an appropriate control - proteins recovered from wells in which no antibody had been immobilised) and used to search protein sequence databases using publicly available software (for example see http://www.mann.embl-heidelberg.de/Services/PeptideSearch). Again BSA was identified as the 1st ranking protein.

Example 6

Generation and inverse screening a monoclonal antibody against a target protein -

bovine serum albumin

To generate monoclonal antibodies to bovine serum albumin (BSA) mice were immunised with the protein by standard methods (see Antibodies, a Laboratory Manual, E. Harlow and D. Lane (Eds.)). Thus, prior to immunisation a pre-bleed was taken from each mouse which was then injected subcutaneously at 2 sites with a 1:1 mixture of protein and Freund's incomplete adjuvant (100µl total) . Twenty one days later tail bleeds were taken and each mouse injected subcutaneously at 2 sites with a further 100µl of a 1:1 mixture of protein and PBS. A further twenty one days later tail bleeds were again taken and each mouse injected subcutaneously at 2 sites with a further 100µl of a 1:1 mixture of protein and PBS. After a further 3 days some mice were splenectomised and a terminal bleed obtained. The splenocytes were extracted from the spleens of suitable mice, fused with myeloma cell line Sp2/0-Ag14 and cultured under selection pressure until the culture supernatants were ready for screening. Prior to screening by the inverse screening process hybridomas were screened by a conventional antibody capture ELISA (see Antibodies, a Laboratory Manual, E. Harlow and D. Lane (Eds.)). Thus, BSA was bound to the wells of 96 well plates by incubation with 0.5-10μg protein/well by standard procedures. Wells were blocked by incubation with 20% soya milk in PBS for 2 hours at room temperature and then washed with PBS before 100μl of hybridoma culture supernatant was applied to each well for 2 hours at room temperature. The wells were washed with PBS before a hybridoma screening anti-mouse Ig-HRP conjugate (Boehringer Mannheim) diluted in 20% soya milk was applied for 2 hours at room temperature. The wells were again washed with PBS before positive wells were visualised by the application of ABTS. It is important to emphasise that this pre-screening step was undertaken for convenience to identify positive hybridomas and hence to avoid unnecessary inverse screening of negative hybridomas. However, with sufficient time and effort or within a high-throughput environment such a pre-screening process is entirely unnecessary.

Wells containing actively growing hybridomas were cloned by limiting dilution and transferred to a serum-free medium. After an appropriate incubation period aliquots of culture supernatant containing antibody were subjected to inverse screening against a mixture of proteins (soya milk proteins containing BSA) as detailed in the previous examples. In this way peptide mass fingerprinting was used to identify those hybridoma clones producing monoclonal antibody to BSA. In some cases the efficacy of the antibody immobilisation and subsequent recovery of BSA from the protein mixture for analysis was improved by precipitation of the antibody from the tissue culture supernatant (using TCA and standard methods) prior to immobilisation. It is also noteworthy that the commercially available serum-free media for the maintenance and propagation of hybridomas were not compatible with direct recovery and immobilisation of antibody from the tissue culture supernatants. This seems most likely to arise from the presence of surfactants within such media preparations that attenuate antibody binding to Immobilon™-P. The invention includes provision for manipulation of the antibodies prior to immobilisation.

Example 7

Generation and inverse screening of monoclonal antibodies against yeast (*S. cerevisiae*) proteins

To generate monoclonal antibodies to *S. cerevisiae* proteins, mice were immunised with the protein by standard methods (see Antibodies, a Laboratory Manual, E. Harlow and D. Lane (Eds.)). Thus, prior to immunisation a pre-bleed was taken from each mouse which was then injected subcutaneously at 2 sites with a 1:1 mixture of protein and Freund's complete adjuvant (100µl total). Twenty one days later tail bleeds were taken and each mouse injected subcutaneously at 2 sites with a further 100µl of a 1:1 mixture of protein and PBS. A further twenty one days later tail bleeds were again taken and each mouse

injected subcutaneously at 2 sites with a further 100µl of a 1:1 mixture of protein and PBS. To establish whether each animal was producing antibodies to a number of different proteins the serum from the various bleeds were used to Western blot yeast proteins separated by 1-DE SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose by standard procedures (see Antibodies, a Laboratory Manual, E. Harlow and D. Lane (Eds.)). The blots were blocked by incubation with 20% soya milk in PBS at 4°C overnight and then incubated with sera from day 0, 21 and 42 bleeds diluted 1:200 - 1:500 in 20% soya milk. Antibody binding to yeast was detected by incubation with anti-mouse Ig conjugated to horse radish peroxidase (Sigma) and the immunoblots developed by enhanced chemiluminescence (ECL, Amersham). The individual animals showed different patterns of antibody reactivity and were producing antibodies to a significant number of proteins (note 1-DE Western blotting will necessary underestimate the potential antibody repertoire of the animals). After a further 3 days some mice were splenectomised and a terminal bleed obtained. The splenocytes were extracted from the spleens and fused with myeloma cell line Sp2/0-Ag14 and cultured under selection pressure until the culture supernatants were ready for screening. Prior to screening by the inverse screening process hybridomas were screened by a conventional antibody capture ELISA (see Antibodies, a Laboratory Manual, E. Harlow and D. Lane (Eds.)). Thus, yeast proteins were bound to the wells of 96 well plates by incubation with 0.5-10μg protein/well by standard procedures. Wells were blocked by incubation with 20% soya milk in PBS for 2 hours at room temperature and then washed with PBS before 100μl of hybridoma culture supernatant was applied to each well for 2 hours at room temperature. The wells were washed with PBS before a hybridoma screening anti-mouse Ig-HRP conjugate (Boehringer-Mannheim) diluted in 20% soya milk was applied for 2 hours at room temperature. Wells containing actively growing hybridomas were cloned by limiting dilution and transferred to a serum-free medium. After an appropriate incubation period aliquots of culture supernatant containing antibody were subjected to inverse screening against a mixture of proteins (yeast proteins)

as detailed in the previous examples. In this way peptide mass fingerprinting was and is being used to identify those hybridoma clones producing monoclonal antibodies to yeast proteins. It is noteworthy, that in this example no prior experimental determination of the peptide mass fingerprint of individual yeast proteins is necessary as the complete genome sequence of *S. cerevisiae* is known and hence the peptide mass fingerprints obtained from the inverse screening process can be compared directly with the theoretical peptide mass fingerprints for all *S. cerevisiae* proteins.

It is important to emphasise that both pre-screening steps (Western blotting and subsequently ELISA) were undertaken for convenience to confirm the potential value of each animal for the production of multiple antibodies and to identify positive hybridomas respectively and hence to avoid unnecessary inverse screening of negative hybridomas. However, with sufficient time and effort or within a high-throughput environment such a pre-screening processes would be entirely unnecessary.

CLAIMS

1. A method of selecting and / or identifying one or more protein affinity ligand's that bind to one or more proteins of interest comprising the steps of:

(A) obtaining a real or theoretical peptide mass fingerprint or other mass spectrometry based characterisation or other protein characterisation of the one or more proteins by either:

i. Subjecting the one or more proteins to peptide mass fingerprinting or other mass spectrometry based characterisation or other protein characterisation; or

ii. Predicting the peptide mass fingerprint or other mass spectrometry based characterisation or other protein characterisation from known data;

(B) utilising the one or more proteins either individually or as a mixture to:

i. Generate one or more antibodies thereto by immunisation ;and/or

ii. Select, using a single or multiple rounds of binding, one or more protein affinity ligands thereto;

(C) screening the one or more antibodies generated in step (B)(i) and/or the one or more protein affinity ligands selected in step (B)(ii) by:

i. adding the one or more proteins individually or as a mixture of proteins to the one or more antibodies generated in step (B)(i) or the one or more protein affinity ligands selected in step (B)(ii), each antibody or protein affinity ligand being used individually, and

ii. after removing any proteins which have not bound, eluting the at least one protein that has bound;

(D) subjecting the at least one eluted protein to peptide mass fingerprinting and / or other mass spectrometry based characterisation and/or other protein characterisation ; and

(E) by comparing the peptide mass fingerprints or other mass spectrometry based characterisation or other protein characterisation obtained in steps (A) and (D) selecting and/or identifying the at least one protein affinity ligand that binds to the one or more proteins of interest.

2. A method as claimed in claim 1 wherein the one or more proteins of interest are resolved by 2D electrophoresis.

3. A method as claimed in claims 1 or 2 wherein between steps (B) and (C) the antibodies obtained in step (B)(i.) are cloned.

4. A method as claimed in any of the preceding claims wherein the one of more proteins of interest are present in a mixture of proteins.

5. A method as claimed in any of the preceding claims wherein the method is a shotgun method for selecting and identifying protein affinity ligands to a plurality of proteins.

6. A method as claimed in any of the preceding claims wherein the other mass spectrometry based characterisation includes acquisition of sequence tag data.

7. A method as claimed in any of the preceding claims wherein the antibodies generated in step (B)(i.) are immobilised on a support comprising

nitrocellulose or PVDF.

8. A method as claimed in claim 7 wherein the support upon which the antibodies are immobilised are treated with an oligosaccharide or polyvinylpyrrolidine solution to block any remaining binding sites.

9. A method as claimed in claim 8 wherein the oligosaccharide is ficoll.

10. A method as claimed in any of claims 7 to 9 wherein the eluting agent is a volatile reagent.

11. A method as claimed in claim 10 wherein the volatile reagent is formic acid.

12. A method of generating monoclonal antibodies to one or more targeted proteins comprising the steps of:

(a) resolving a complex protein mixture;

(b) subjecting the resolved protein(s) to peptide mass fingerprinting to obtain a peptide mass profile or obtain a theoretical peptide mass profile;

(c) utilising one or more of the resolved proteins to generate one or more monoclonal antibodies thereto;

(d) adding the or another complex protein mixture to the one or more monoclonal antibodies generated in Step (c), to select those proteins which bind the one or more monoclonal antibodies, and subjecting the selected protein(s) to peptide mass fingerprinting to obtain a peptide mass profile;

(e) comparing the peptide mass profiles obtained in steps (b) and (d); and

(f) selecting one or more hybridoma clones of interest.

13. A method of generating an antibody library comprising the steps of:

(a) resolving a complex protein mixture and subjecting the resolved protein(s) to peptide mass finger printing to obtain a peptide mass profile; or

(b) obtaining a theoretical peptide mass profile for a protein which is sought;

(c) utilising the or another complex protein mixture to generate one or more monoclonal antibodies thereto;

(d) adding the or the other complex protein mixture to the one or more monoclonal antibodies generated in Step (c) to select those proteins which bind the one or more monoclonal antibodies, and subjecting the selected protein(s) to peptide mass fingerprinting to obtain a peptide mass profile;

(e) comparing the peptide mass profiles obtained in steps(a or b) and (d); and

(f) identifying the monoclonal antibodies of potential interest for a monoclonal antibody library.

14. A process for selecting desired members of an affinity ligand library comprising the steps of:

(a) resolving a complex protein mixture and subjecting the resolved protein(s) to peptide mass finger printing to obtain a peptide mass profile; or

(b) obtaining a theoretical peptide mass profile for a protein which is sought;

(c) utilising one or more of the resolved proteins to select one or more affinity ligands from a library;

(d) adding the or another complex protein mixture to the one or more affinity

ligands generated in step (c) to select those proteins which bind the one or more affinity ligands, and subjecting the selected protein(s) to peptide mass fingerprinting to obtain a peptide mass profile;

(e) comparing the peptide mass profiles obtained in steps (a or b) and (d); and

(f) selecting one or more affinity ligands of interest.

15. A method of screening an affinity ligand to a protein characterised in that the affinity ligand is generated or selected using an impure protein or a complex protein mixture and then identified by comparing a peptide mass fingerprint or other mass spectrometry based characterisation or other protein characterisation obtained from the protein/proteins for which it is specific with that of a peptide mass fingerprint or other mass spectrometry based characterisation or other protein characterisation which is theoretical for said protein/proteins or is obtained from the impure protein or complex protein mixture.

16. A method of selecting an antibody of other protein affinity ligand specific to a given peptide characterised in that the antibody or other protein affinity ligand is selected by comparing a peptide mass fingerprint or other mass spectrometry based characterisation or other protein characterisation of the protein/proteins released from the antibody or other protein affinity ligand to which it binds with a peptide mass fingerprint or other mass spectrometry based characterisation or other protein characterisation which is theoretical for said protein/proteins or is obtained from the known protein.

17. A method as claimed in any of the preceding claims wherein the peptide mass fingerprint is obtained by mass spectrometry.

18. A method as claimed in any of the preceding claims further comprising the use of automated well plate handling technology and automated high-throughput mass spectrometry.

Complex Protein Mixture
(A)
2-D Gel(s)
(Stained to Reveal Proteins)
Individual Proteins of Interest Obtained by Any Method
Proteins of Interest
(B - optional)
Peptide Mass Fingerprint
(C)
Immunise Animal
Initial Screen by Micro Dot Blot - to Confirm Immune Response - Not Essential to Process
Fuse Spleen Cells with Myeloma Cell Line
Hybridomas
Clone by Limiting Dilution
Remove Tissue Culture Medium
Affinity Purify Antibodies from Medium
Characteristic Peptide Mass Fingerprint
(E)
Select Hybridoma Clones
(F)
Match Profiles to Profile of Protein of Interest
Peptide Mass Fingerprint Released Proteins
(D)
Release Proteins from Antibodies
Add Complex Protein Mixture
Immobilise Antibodies (96 Well Plate or Other Format)
Antibody 1 (MAb1)
Antibody 2 (MAb2)
Antibody 3 (MAb3)
etc.

FIG. 1.

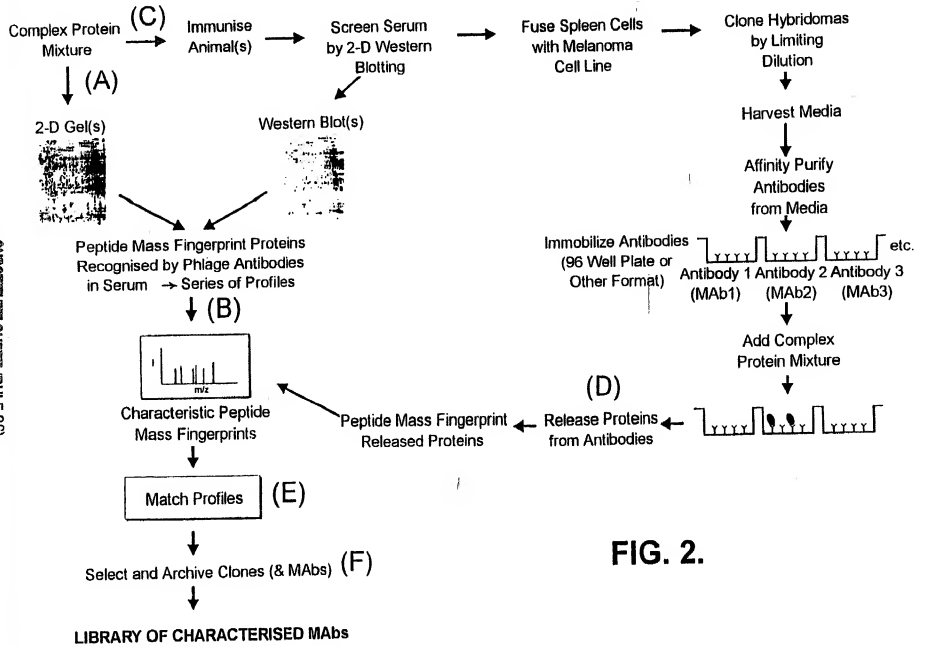


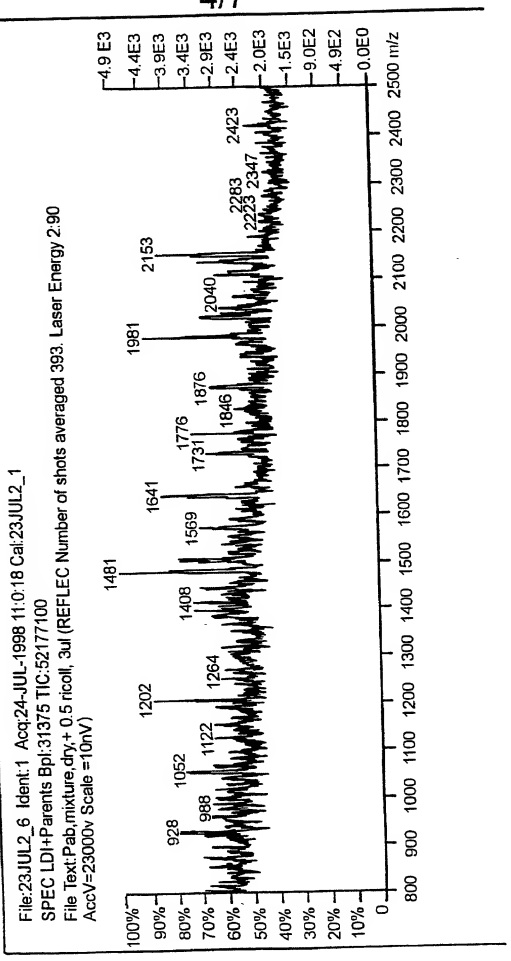
FIG. 2.

09/582971
PCT/GB99/00071

Complex Protein Mixture
(A)
2-D Gel(s)
(B)
Peptide Mass Fingerprint Proteins Recognised by Phage Antibodies
→Series of Profiles
Phage Displayed Antibody Library (or Other Library)
(C)
Western Blots)
Recover Spots & Bound Phage
Use Phage to Re-infect E-coli (Host)
Select Individual Clones of Host & Grow Up to Produce Phage
Isolate Phage Displayed Antibodies (PhAbs)
Antibody 1 (PhAb1)
Antibody 2 (PhAb2)
Antibody 3 (PhAb3)
etc.
Add Complex Protein Mixture
Immobilize (PhAbs)
(D)
Elute Bound Proteins
Peptide Mass Fingerprint Released Proteins
m/z
Characteristic Peptide Mass Fingerprints
Match Profiles
(E)
Select Clones Harbouring Relevant Phage Archive Clones (& PhAbs)
(F)
LIBRARY OF CHARACTERISED PhAbs

FIG. 3.

FIG. 4A.


File:23JUL2_6 Ident:1 Acq:24-JUL-1998 11:0:18 Cal:23JUL2_1
SPEC LDI+Parents Bpl:31375 TIC:52177100
File Text:Pab,mixture,dry,+ 0.5 ricoll, 3ul (REFLEC Number of shots averaged 393. Laser Energy 2:90
AccV=23000v Scale =10nV)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0
928
988
1052
1122
1202
1264
1408
1481
1569
1641
1731
1776
1846
1876
1981
2040
2153
2223
2283
2347
2423
800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900 2000 2100 2200 2300 2400 2500 m/z
4.9 E3
4.4E3
3.9E3
3.4E3
2.9E3
2.4E3
2.0E3
1.5E3
9.0E2
4.9E2
0.0E0

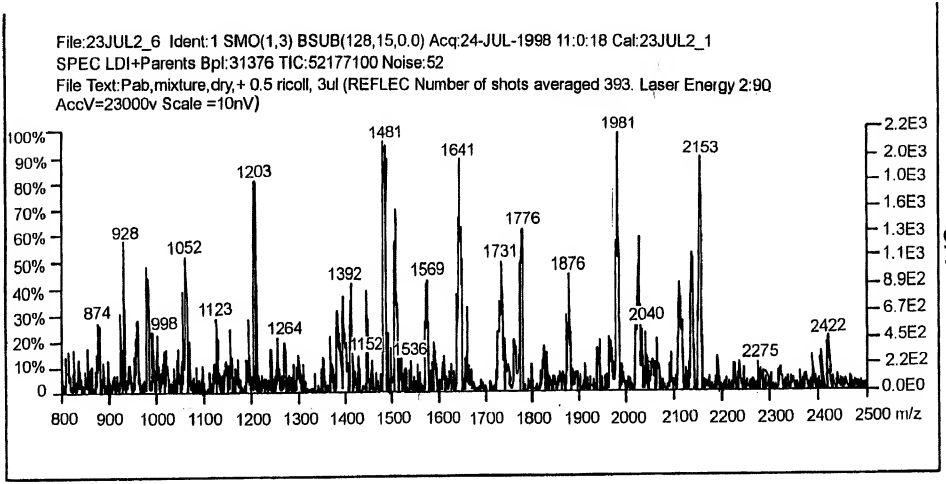
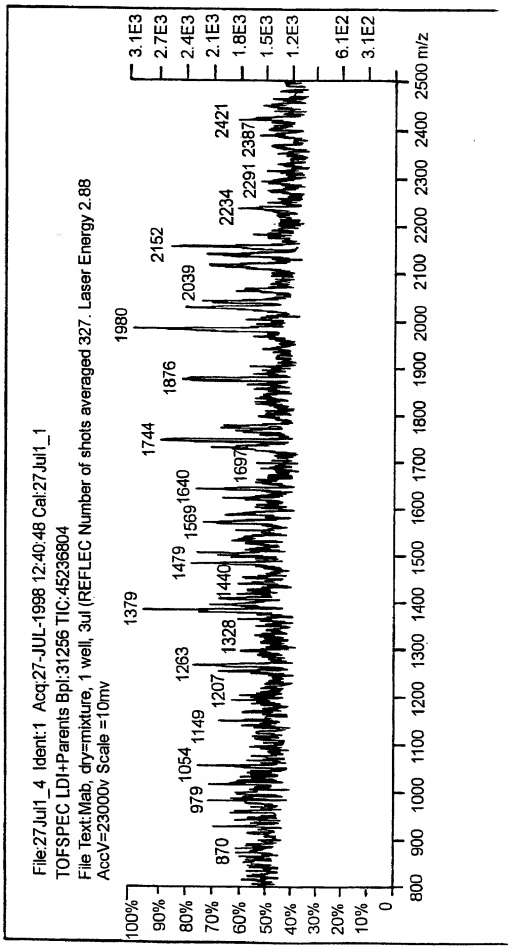


FIG. 4B.

FIG. 5A.


File:27Jul1_4 Ident:1 Acq:27-JUL-1998 12:40:48 Cal:27Jul1_1
TOFSPEC LDI+Parents Bpl:31256 TIC:45236804
File Text:Mab. dry=mixture, 1 well, 3ul (REFLEC Number of shots averaged 327. Laser Energy 2.88
AccV=23000v Scale =10mv
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0
800 900 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900 2000 2100 2200 2300 2400 2500 m/z
3.1E3
2.7E3
2.4E3
2.1E3
1.8E3
1.5E3
1.2E3
6.1E2
3.1E2
870
979
1054
1149
1207
1263
1328
1379
1440
1479
1569
1640
1697
1744
1876
1980
2039
2152
2234
2291
2387
2421

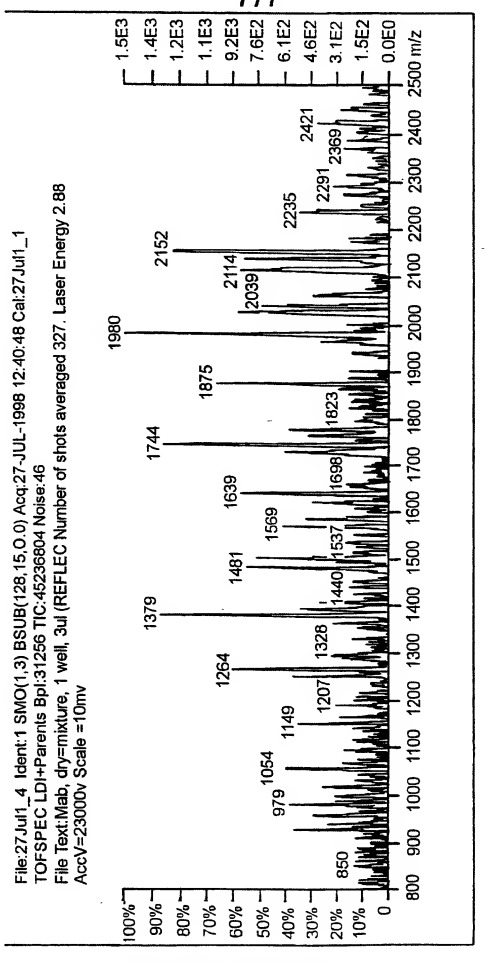

File:27Jul1_4 Ident:1 SMO(1,3) BSUB(128,15,0.0) Acq:27-JUL-1998 12:40:48 Cal:27Jul1_1
TOFSPEC LDI+Parents Bpl:31256 TIC:45236804 Noise:46
File Text:Mab, dry=mixture, 1 well, 3ul (REFLEC Number of shots averaged 327. Laser Energy 2.88
AccV=23000v Scale =10mv
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0
800 850 900 979 1000 1054 1100 1149 1200 1207 1264 1300 1326 1379 1400 1440 1481 1500 1537 1569 1600 1639 1698 1700 1744 1800 1823 1875 1900 1980 2000 2039 2100 2114 2152 2200 2235 2291 2300 2369 2400 2421 2500 m/z
1.5E3
1.4E3
1.2E3
1.1E3
9.2E3
7.6E2
6.1E2
4.6E2
3.1E2
1.5E2
0.0E0

FIG. 5B.

Docket No. 60,319-010 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Stephen Roy Pennington

Serial No. Filed herewith

For: USE OF MASS FINGERPRINTING FOR IDENTIFICATION OF PROTEIN AFFINITY LIGANDS

Assistant Commissioner For Patents
Washington, D.C. 20231

COMBINED DECLARATION AND POWER OF ATTORNEY
(Entry into the National Phase of an International Application in the USA)

I, the undersigned inventor, hereby declare that:

My residence, post office address, and citizenship are as stated next to my name below;

I believe that I am the first and original inventor of the subject matter claimed in the application for patent entitled USE OF MASS FINGERPRINTING FOR IDENTIFICATION OF PROTEIN AFFINITY LIGANDS which is described and claimed in the U.S. Patent Application filed herewith;

I have reviewed and understand the contents of the above-identified application for patent (hereinafter "the application"), including the claims;

I acknowledge the duty under Title 37, Code of Federal Regulations, Section 1.56(a), to disclose information known to be material to the patentability of this application. I also acknowledge that information is material to patentability when it is not cumulative to information already provided to the United States Patent and Trademark Office and when it either compels, by itself or in combination with other information, a conclusion that a claim is unpatentable under the preponderance of evidence standard, before any consideration is given to evidence which may be submitted to establish a contrary conclusion of patentability, or refutes or is inconsistent with a position taken in either (i) asserting an argument of patentability, or (ii) opposing an argument of unpatentability relied on by the United States Patent and Trademark Office;

I hereby claim the priority benefit under Title 35, Section 365(c), of the following PCT International Patent Application designating the United States:

Application No.	Filing Date	Based on
PCT/GB99/00071	08 January 1999	UK Patent Application 9800378.3 (filed on 08 January 1998)

Where the subject matter of the claims of this application is not disclosed in the priority PCT International Application, we acknowledge the duty to disclose information known to be material to the patentability of this application that became available between the filing dates of this application and of the priority PCT International Application;

I hereby appoint as attorneys with full power of substitution to prosecute this application and conduct all business in the United States Patent and Trademark Office associated with this application the firm of DYKEMA GOSSETT PLLC, including Charles R. Rutherford, Reg. No. 18,933, Robert L. Kelly, Reg. No. 31,843, Ernest E. Helms, Reg. No. 29,721, Kevin M. Hinman, Reg. No. 35,193, William F. Kolakowski III, Reg. No. 41,908, and John F. Buckert, Reg. No. 44,572, located at 39577 Woodward Avenue, Suite 300, Bloomfield Hills, Michigan 48304.

6

declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Full Name of inventor: Stephen Roy Pennington

Inventor's Signature:

Post Office and Residence: UNIVERSITY OF LIVERPOOL, L69 3BX, U.K. GB

Citizenship: BRITISH Date: 4th July 2000

BH\258621
IDV RLK:dc